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Control of somatic embryogenesis and embryo development by AP2 transcription factors

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Abstract Members of the AP2 family of transcription factors, such as *BABY BOOM* (*BBM*), play important roles in cell proliferation and embryogenesis in *Arabidopsis thaliana* (*AtBBM*) and *Brassica napus* (*BnBBM*) but how this occurs is not understood. We have isolated three *AP2* genes (*GmBBM1, GmAIL5, GmPLT2*) from somatic embryo cultures of soybean, *Glycine max* (L.) Merr, and discovered *GmBBM1* to be homologous to *AtBBM* and *BnBBM*. *GmAIL5* and *GmPLT2* were homologous to Arabidopsis *AINTEGUMENTA-like5* (*AIL5*) and *PLETHORA2* (*PLT2*), respectively. Constitutive expression of *GmBBM1* in Arabidopsis induced somatic embryos on vegetative organs and other pleiotropic effects on post-germinative vegetative organ development. Sequence comparisons of BBM orthologues revealed the presence of ten sequence motifs outside of the AP2 DNA-binding domains. One of the motifs, bbm-1, was specific to the *BBM*-like genes. Deletion and domain swap analyses revealed that bbm-1 was important for somatic embryogenesis and acted cooperatively with at least one other motif, euANT2, in the regulation of somatic embryogenesis and embryo development in transgenic Arabidopsis. The results provide new insights into the mechanisms by which BBM governs embryogenesis.

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Introduction

BABY BOOM (*BBM*) is a member of the AP2 family of transcription factors which have diverse functions in plant development (Nole-Wilson et al. 2005; Floyd and Bowman 2007; Feng et al. 2005). The AP2 family belongs to the AP2/ERF superfamily. This is one of the largest groups of plant transcription factors and has undergone extensive duplication and domain shuffling during its evolution (Riechmann et al. 2000; Kim et al. 2006; Nakano et al. 2006). Members have double AP2/ERF domains in the AP2 family, single AP2/ERF DNA-binding domains in the ERF family and single AP2/ERF domains together with a B3 DNA-binding domain in the RAV family (Riechmann and Meyerowitz 1998). The superfamily consists of 147 members in Arabidopsis, 157 members in rice (Nakano et al. 2006) and 148 members in soybean (Zhang et al. 2008). Members function in diverse processes fundamental to plant growth, reproduction and environmental interactions (Riechmann and Meyerowitz 1998; Feng et al. 2005; Nole-Wilson et al. 2005). In *Arabidopsis*, rice and soybean (Sakuma et al. 2002; Gong et al. 2004; Nakano et al. 2006; Zhang et al. 2008) the members can be grouped by sequence similarity into the same family and subfamily groupings (Floyd and Bowman 2007). *BBM* clusters within one of the sublineages, euANT, which appears to have specialized in meristem differentiation and maintenance (Floyd and Bowman 2007).

Functional studies of the AP2 family members, such as *APETALA2 (AP2), AINTEGUMENTA (ANT), BABY BOOM (BBM), PLETHORA1 (PLT1), PLETHORA2 (PLT2)* and the *AINTEGUMENTA-like (AIL)* genes, have revealed diverse transcriptional networks and developmental processes that the family controls as well as redundancies that exists among and within the different groups. *APETALA 2* (*AP2*), the first member of the family to be reported, functions independently in the specification of floral organ identity (Jofuku et al. 1994; Okamuro et al. 1997, Maes 1999) and in the maintenance of the stem cell niche of the shoot meristem (Würschum et al. 2006). *AINTEGUMENTA (ANT)* is required for ovule development and floral organ growth (Elliott et al. 1996, Klucher et al. 1996). *ANT* can act redundantly with *AP2* in floral development (Krizek et al. 2000). *BABY BOOM (BBM)* has been implicated in the differentiation of embryonal stem cells from somatic cells (Boutilier et al. 2002) and clusters within the same clade as *PLETHORA* (*PLT1* and *PLT2*) which controls root stem cell identity and maintenance (Aida et al. 2004). *PLT1, PLT2, BBM* and *PLT3/AIL6* function redundantly in root meristem and embryo differentiation (Galinha et al. 2007). They are also closely related to a number of other *AINTEGUMENTA*-like (*AIL*) genes (Nole-Wilson et al. 2005; Tsuwamoto et al. 2010) which are generally involved in the specification of meristems or division-competent states (Nole-Wilson et al. 2005).

Members of the AP2 family share two highly-conserved AP2 DNA-binding domain repeats separated by a linker region; however, the N-terminal and C-terminal sequences are very distinct. The domains within these regions have not been studied but they are likely to be important for the specific transcriptional activities, protein interactions and nuclear localizations that confer the unique functions associated with each member (Nakano et al. 2006). Sequence comparisons of the AP2/ERF superfamily members from soybean, Arabidopsis and rice have revealed the presence of many conserved motifs outside of the AP2/ERF DNA binding domain raising the possibility that shared conserved motifs may form the basis for functional similarities among different groups (Zhang et al. 2008).

BABY BOOM (*BnBBM*) was cloned from *Brassica napus* microspore embryo cultures and was shown to induce somatic embryos when ectopically overexpressed in *Arabidopsis* or *B. napus* (Boutilier et al. 2002). The acquisition of totipotency through this process was accompanied by a number of pleiotropic effects on plant development (Boutilier et al. 2002). In transgenic tobacco, *BnBBM* expression induced pleiotropic effects on vegetative growth and development but did not induce embryogenesis (Srinivasan et al. 2007) indicating that embryogenic pathways differ among species or that the domains within BBM that govern embryogenesis have diverged in sequence among plants and were not recognized. It is currently believed that *BnBBM* enhances cell proliferations that can result in different developmental outcomes including organogenesis or embryogenesis (Srinivasan et al. 2007). The variety of different pleiotropic effects on plant development that were observed with ectopically-expressed *BnBBM* may indicate broad redundancies among AP2 family

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members beyond those already demonstrated with the *PLT*-like and *BBM* genes (Galinha et al. 2007) or the *AP2* and *ANT* genes (Krizek et al. 2000).

In this study we examined *BBM* orthologues from a non-cruciferous species, soybean, to determine if the same developmental pathways were induced in transgenic plants; to identify conserved motifs in *BBM*-like genes; and to locate the determinants of embryogenesis. *GmBBM1* was identified as the functional orthologue of *AtBBM* and *BnBBM* through both structural and functional studies. The modular domain structure of *BBM*-like genes was also analyzed and revealed motifs that were important for their specificity in the induction of somatic embryogenesis.

Materials and methods

Plant material

Embryos were isolated from soybean, *Glycine max* (L.) Merril genotype X5, formerly called X2650-7-2-3 (Simmonds and Donaldson 2000). Donor plants were grown under a 16 h photoperiod in the greenhouse as previously reported (Simmonds and Donaldson 2000). Embryogenic cultures were established as previously described (Finer 1988; Finer and Nagasawa 1988). Immature cotyledons, 4 and 5.9 mm in size, were placed abaxial side-down on MSD40 medium (Finer and Nagasawa 1988) containing MS salts, B5 vitamins, 6%(w:v) sucrose, 40 mg/l 2,4-D and 0.2% Gelrite, pH 5.8 and cultured at 27°C under a photoperiod of 16 h with a light intensity of 60–70 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. Secondary globular embryos developing on the cultured cotyledons were transferred to 125 ml Erlenmeyer flasks with 30 ml of 10A40N medium (Finer and Nagasawa 1988) containing modified MS salts (with MS nitrogen replaced by 10 mM NH4NO3 and 30 mM KNO3), B5 vitamins, 6% sucrose, 5 mg/l 2,4-D, and 5 mM asparagine, pH 5.8 and cultured as above at 30 μmol m^{-2} s^{-1} on a rotary shaker at 125 rpm. Embryogenic tissue (30–75 mg) was subcultured to fresh medium every 2–4 weeks to maintain proliferation. Proliferation was stopped and embryo maturation was initiated by culturing embryogenic tissue for 4 weeks on solid OMSM6 medium without charcoal under a light intensity of 70 μmol m^{-2} s^{-1}. Embryos were desiccated in 85% relative humidity and germinated on B5 medium with 3% sucrose and 0.6% Phytagar or 0.2% Gelrite, pH 5.8. Plants with two trifoliate leaves were transplanted to soil and grown under a 16-h photoperiod prior to transfer to a 12-h photoperiod for seed formation.

Root tissues were harvested from 1-week old seedlings. Leaf and stem tissues were from 3-weeks old seedlings. Flowers were harvested 2 days after full anthesis. Developing seeds and pods were harvested from different stages between 6 and 24 days post anthesis (dpa) at 3 days intervals. To study the expression of *GmBBM1* genes in developing seeds tissues from seed coat, embryo and pods were collected separately and RNA was extracted using the RNeasy Kit (Qiagen, Canada). Tissues from soybean roots, leaves, stems and flowers were also collected, homogenized in liquid nitrogen and total RNA was extracted using Trizol (Invitrogen, Canada).

RT-PCR

Probes generated by RT-PCR were used for library screening and expression profiling. Primers specific to each of the three soybean genes, *GmBBM1*, *GmPLT2* and *GmAIL5*, are shown in Supplementary Table 1. The SoyTub2 gene was used as positive control. PCR conditions for *GmPLT2* and *GmAIL5* were as follows: 3 min at 95°C; 30 cycles [1 min 95°C, 1 min specific annealing temperature (Supplementary Table 1), 1 min 72°C] and 5 min extension at 72°C. PCR conditions for *GmBBM1* and SoyTub2 were as follows: 31 min at 50°C, 15 min at 95°C; 30 cycles (30 s 94°C, 30 s specific annealing temperature (Supplementary Table 1), 30 s 72°C) and 2 min extension at 72°C.

Library construction

Total RNA (2 mg/g fresh tissue) was extracted from cultured embryogenic soybean suspension cultures and mRNA (0.2% of total RNA) was purified using the Quik mRNA Isolation Kit (Stratagene/VWR, Canada). A cDNA library was constructed using Lambda-ZAPII cDNA synthesis kit (Stratagene/VWR, Canada). Phages (1.6×10^6pfu) were plated according to the manufacturer's instructions. The amplified cDNA library titre was 2.5×10^9pfu. The cDNA library phage plaques were hybridized using a probe containing the AP2 domain and linker using primers designed from the alignment of EST *AW200688* coding for a soybean AP2 protein and *BnBBM1* from *Brassica napus* as described in Results. cDNA were excised from the lambda-ZAPII vectors following the conversion of the lambda-ZAP clone plasmids pBluescript SK DNA. Sequences were determined with the ABI PRISM TM dye terminator cycle sequencing kit (PE Applied Biosystems). Data was analyzed using DNAsis (Hitachi Corporation 2003) Genejumper primer insertion kit for sequencing version B (Invitrogen, Canada) was used in order to be able to sequence the full-length clones.

Arabidopsis transformation and assessment

Plant transformations were carried out according to Clough and Bent (1998). *GmBBM1* was cloned into the pBINPLUS vector and expression was driven by the double 35S

promoter and alfalfa mosaic virus (AMV) translational enhancer as described in Boutilier et al. (2002). Selection was performed on 50 mg/l kanamycin. Thousands of seeds were screened until a collection of approximately 35 lines with somatic embryos were recovered. Of these, six representative lines were studied in greater detail over successive generations.

BnBBM1 mutants were generated by inverse PCR reactions on the *BnBBM1* PUC19 plasmid, using Expand Long Template PCR System (Roche) and synthetic primers with 10-bp sequence including a BamHI or XhoI sites in their 5′-ends that replaces the original sequences. The following primers were used for euANT2 (N1BamHIF 5′-CGGGATCCCTGAGAAATCAACCCGTGGATAATG-3′ and N1BamHIR5′-CTGGATCCGCCACCACCACCGTCT CCTCCTC-3′) and for bbm-1 (N2XhoIF5′-CGGCTCGA GCCTTATGAACAAAATCACCATCG-3′ and N2XhoI R5′-CCGCTCGAGGGTGGAAGTATTTGAAAGAAAT-3′). These constructs were obtained by KpnI/SalI digestion and cloned into the binary vector pCAMBIA1300, along with the wild-type *BnBBM1* gene as the control. They were introduced into *Agrobacterium tumefaciens* GV3101 for plant transformation and selection was performed with 30 mg/L hygromycin. Approximately, 3,000–4,000 seeds in total were screened in triplicate experiments to assess the phenotypes of the transgenic lines.

For the domain swap analysis, the PLT2, AIL5, and AIL7 genes from *Arabidopsis* were synthesized with the BnBBM1 bbm-1 motif (SLGLSMIKTWLRNQP) as shown in Fig. 2A by GenScript Corporation (Piscataway, NJ). They were cloned into pCAMBIA1300 under control of the double 35S promoter. As above the constructs were introduced into *Agrobacterium tumefaciens* GV3103 for plant transformation and screened.

Seeds from each transgenic seed line were sterilized in a solution of 70% ethanol for 30 s followed by a solution of 25% javex plus 0.05% Triton for 20–30 min. The seeds were shaken for the duration. They were then rinsed 4 times with sterile distilled water and placed in a 0.1% solution of agarose at 4°C overnight. The next day, the seeds were plated on ½ MSB5 media (Sigma Chemical Co) with 3% sucrose and 0.6% agarose along with selectable agents: 50 mg/l kanamycin for pBINPLUS or 30 mg/l hygromycin for pCAMBIA1300. Seedlings were then surveyed for phenotype over a 2–3 week period. At 3–4 weeks growth they were transferred to soil and brought to flowering stage.

Phylogenetic analysis

Sequences of double AP2 domain proteins similar to *AtBBM* were mined from GenBank® (National Institutes of Health genetic sequence database at National Center for Biotechnology Information) using the Entrez browser (available at: http://www.ncbi.nlm.nih.gov/) and from the TIGR Gene Indices. Proteins that were not full-length were not included as we intended to eventually assess the N-terminal and the C-terminal sequences also. To identify those sequences belonging to the euANT subgroup, sequences were screened for the 10 amino acid insertion in the first AP2 domain characteristic of this group as well as a predomain region longer than 127 amino acids, as described by Kim et al. (2006). *WRI1* from the basalANT subgroup, a separate ANT lineage, was included as an outgroup. The mined amino acid sequences were aligned using CLUSTALW (Thompson et al. 1994) and further improved by visual examination and editing using GeneDoc© Version 2.6.002 (Nicholas and Nicholas 1997). The AP2 domain was used for alignment as the full length sequences were too divergent to successfully align all the sequences retrieved from GeneBank® and TIGR Gene Indices database. We limited the boundaries of the double AP2 domain to the sequences starting 5 amino acids upstream and approximately 10 amino acids downstream of the conserved double AP2 domains. In total, 53 sequences (Supplementary Table 2) were analyzed.

The aligned sequences were first subjected to a tool for the selection of the best fit model from among 112 models of protein evolution, using ProtTest (Abascal et al. 2005) version 2.2. After the likelihood statistics were completed three statistical frameworks were selected consecutively one at a time to determine which candidate model fits the data best. Although a tree using the model-averaged estimate of the parameters was obtained in the analysis and examined, we carried a separate Maximum Likelihood (ML) phylogenetic analysis using the PROML program (PROtein Maximum Likelihood program) in the PHYLIP package (Felsenstein 2008) with the selected evolutionary model along with its specific parameters, previously obtained from ProtTest. In the analysis 100 bootstrap trees were produced from which a majority rule tree was computed to obtain bootstrap support for the branches on the ML tree and a strict consensus tree was also computed for examination. Another similar bootstrap analysis was carried with MEGA4 (Nei and Kumar 2000) with 1,000 repeats.

Conserved motifs were identified through a combination of ClustalW alignment, MEME version 4.0.0 (Bailey and Elkan 1994), and Block Maker (Henikoff et al. 1995). The Eukaryotic Linear Motif resource for Functional Sites in Protein (http://elm.eu.org/links.html) was used to predict motif function.

Results

AP2 family genes cloned from soybean somatic embryos

Soybean cDNAs with sequences similar to *BnBBM1*, were isolated from immature somatic embryos of soybean, *G. max* cv Merril genotype X5 (Simmonds and Donaldson 2000). The probe used to screen the cDNA library was strategically designed using PCR primers (Supplementary Table 1) targeted to sequences identified from alignments of *BnBBM1* and a partial soybean putative AP2 protein *EST AW200688* from a cDNA library constructed from cotyledons of 3- and 7-day-old seedlings of cultivar Williams (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucest&val=6481417) (Shoemaker R, The Public Soybean EST Project) (Biogenetics Services, SD, USA) sequences.

Two clones, *GmBBM1* (2.5 kb, 707 aa, GenBank Accession HM775856) and *GmAIL5* (2.3 kb, 558 aa, GenBank Accession HM775857), were identified and isolated. Another cDNA clone (2 Kb, 560 aa), *GmPLT2,* was first identified as two 5′-truncted cDNA clones that later appeared as a full length clone (EU677381). All possessed high levels of sequence similarity in the double AP2 domain region (97 %) which decreased in the N- and C-terminal regions (Supplementary Fig. 1A). The soybean *GmBBM1* sequence was similar to those of the cruciferous genes, *BnBBM1* and *AtBBM,* with more than 91% similarity in the double AP2 domains (Supplementary Fig. 1B). This decreased in the N- and C-terminal sequences to 48 and 30% respectively (Supplementary Fig. 1B).

Phylogenetic analysis of *GmBBM1, GmAIL5 and GmPLT1*

To examine the relationships of the three soybean genes with other AP2 family members all available genes in The Arabidopsis Information Resource (http://www.arabidopsis.org/), GenBank, DFCI (Dana Farber Cancer Institute) Gene Index (http://compbio.dfci.harvard.edu/tgi/; formerly TIGR gene index), and the TIGR Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu) at the time of the study with sequence similarity to *BBM* genes were retrieved using the double AP2 domain region of the *AtBBM* sequence. Only full-length sequences and those with at least 60% similarity in the double AP2 domain were retained. Sequences belonging to the euANT subgroup are characterized by the presence of a 10 amino acid insert in the first AP2 domain region and a relatively long predomain region (Kim et al. 2006). These two characteristics were used to select 49 sequences belonging to the euANT subgroup. *WRI1*, belonging to the basalANT subgroup, was included as an outgroup. A total of 53 sequences (Supplementary Table 2) were included in the phylogenetic analysis. The alignment was carried out using the region spanning the two AP2 domains, including the linker region, starting 5 residues upstream of the first conserved AP2 domain and finishing approximately 10 amino acids downstream of the second AP2 domain. The evolutionary model that best fit this data was the JTT + G model (i.e. the Jones-Taylor-Thornton model) under the three statistical frameworks analyzed, i.e. AIC, AICc and BIC, and its parameter G = 0.558, i.e. gamma shape with 4 rate categories.

Figure 1 shows the relationship of the three soybean genes to the euANT subgroup based on the AP2-linker-AP2 sequences. *GmBBM1* is closest to the other legume *BBM* gene, *MtBBM*, from *Medicago truncatula* and also clusters closely with the *BBM* genes from the *Brassicacea* and *Poaceae* families. *GmAIL5* is grouped with the oil palm *EgAp2-1* gene and is close to *AIL5* from *Arabidopsis*. *GmPLT2* is grouped with the *Arabidopsis PLT1* and *PLT2* genes along with a gene from *Vitis vinifera*, all of which form the larger *PLT*-like grouping with *Arabidopsis AIL6* and *AIL7* and sequences from rice, *Brassica rapa*, and another sequence from *Vitis vinifera*.

Motif composition and identification of a *BBM*-specific motif

To further distinguish the genes in the *BBM*-like grouping from other euANT genes, the more variable N-terminal and C-terminal protein sequences were aligned to search for conserved motifs (see Supplementary Fig. 1C). In the N-terminal sequences, five motifs were identified in most members of the *BBM*-like grouping. Three of these motifs were previously identified in the euANT lineage (euANT2, 3, and 4; Kim et al. 2006) and were found throughout most of the sequences analyzed (Fig. 2A). The fourth motif (bbm-1) was specific to the *BBM*-like genes and absent from the closely-related *PLT*-like genes (Fig. 2A). This motif was weakly conserved in some of the *ANT*-like genes, particularly *GbANTL1*, *PtANTL1*, and CAO45633. It was also found in Os01g67410, which was not associated with any of the identified groupings. The fifth motif (euANT5) was also identified in the majority of the members of the *BBM*-like grouping but was absent in the *BBM*-like genes from *Cenchrus ciliaris* and *Pennisetum squamulatum* (Fig. 2A). This motif is also conserved in the *ANT*-like genes as well as the *AIL1*-like genes, and representatives containing this motif can also be found amongst the *PLT*-like genes, although it is completely absent from the *AIL5*-like genes. Interestingly the bbm-1 and euANT5 motifs bore some similarity, both including the sequence LSM.

In the C-terminal sequences five other motifs were identified (Fig. 2B). A motif (euANT6) was identified in all

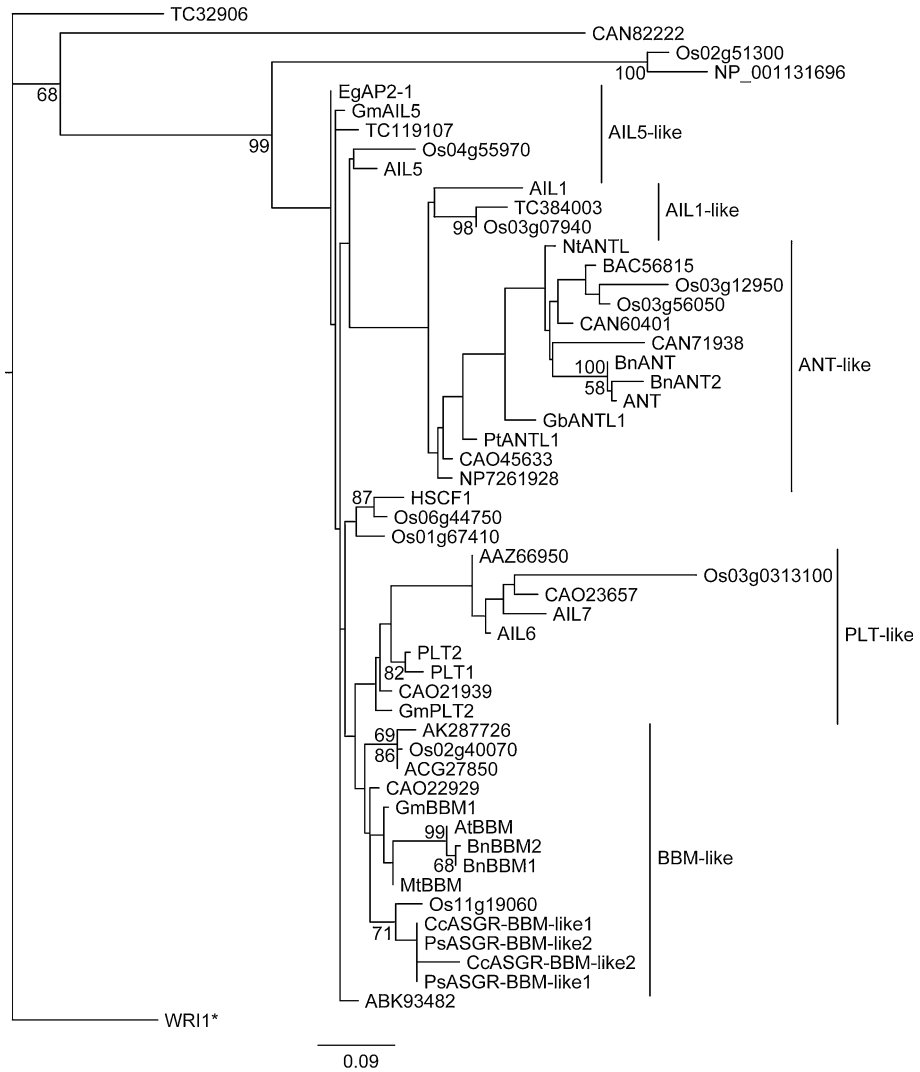

Fig. 1 Maximum likelihood tree of the euANT subgroup of the double AP2 family

of the *BBM*-like genes immediately following the second AP2 domain. It was identified in representatives from all of the major euANT lineages, although the degree of conservation varied considerably (Fig. 2B). This motif contains a number of Lys and Arg residues, suggesting that it may at least partially function as a nuclear localization signal, as has been suggested for the *PLT* genes (Aida et al. 2004). The four additional motifs in the C-terminal sequences of the *BBM*-like genes were conserved with a low degree of specificity. The bbm-2 motif was only weakly conserved in the *Arabidopsis* and *B. napus BBM* genes and bbm-3 was completely absent. Some of these motifs were weakly conserved in sequences outside of the *BBM*-like grouping particularly bbm-3 and bbm-4 in some of the *PLT*-like sequences. As with the bbm-1 motifs, Os01g67410 also contained the bbm-2 and bbm-4 motifs, although the bbm-3 motif was only weakly conserved and the bbm-5 motif was absent.

Several of the motifs, including euANT2, bbm-1, euANT5, and euANT6, contain consensus sequences for phosphorylation. euANT5 also contains a consensus sequence for sumoylation. The euANT2 motif also conforms to a TRFH domain docking motif and a WW ligand motif.

Expression profile of *GmBBM1*, *GmAIL5* and *GmPLT1*

In soybean plants, the expression of *GmBBM1* paralleled that of *AtBBM* in Arabidopsis (Boutilier et al. 2002). It was selectively expressed in soybean embryos and young roots (Fig. 3). *GmAIL5* also possessed the same expression pattern as *AIL5* in Arabidopsis (Nole-Wilson et al. 2005). It was expressed in all of the soybean organs undergoing growth and development that were examined (Fig. 3). *GmPLT2* had the same expression pattern as *PLT1* and *PLT2* in Arabidopsis (Aida et al. 2004) and *M. truncatula* (Imin et al. 2007; Holmes et al. 2008). It was expressed predominantly in soybean roots and to a lesser extent in soybean embryos (Fig. 3). The data supported the assignment of *GmBBM1* as a BBM homologue, *GmAIL5* as an

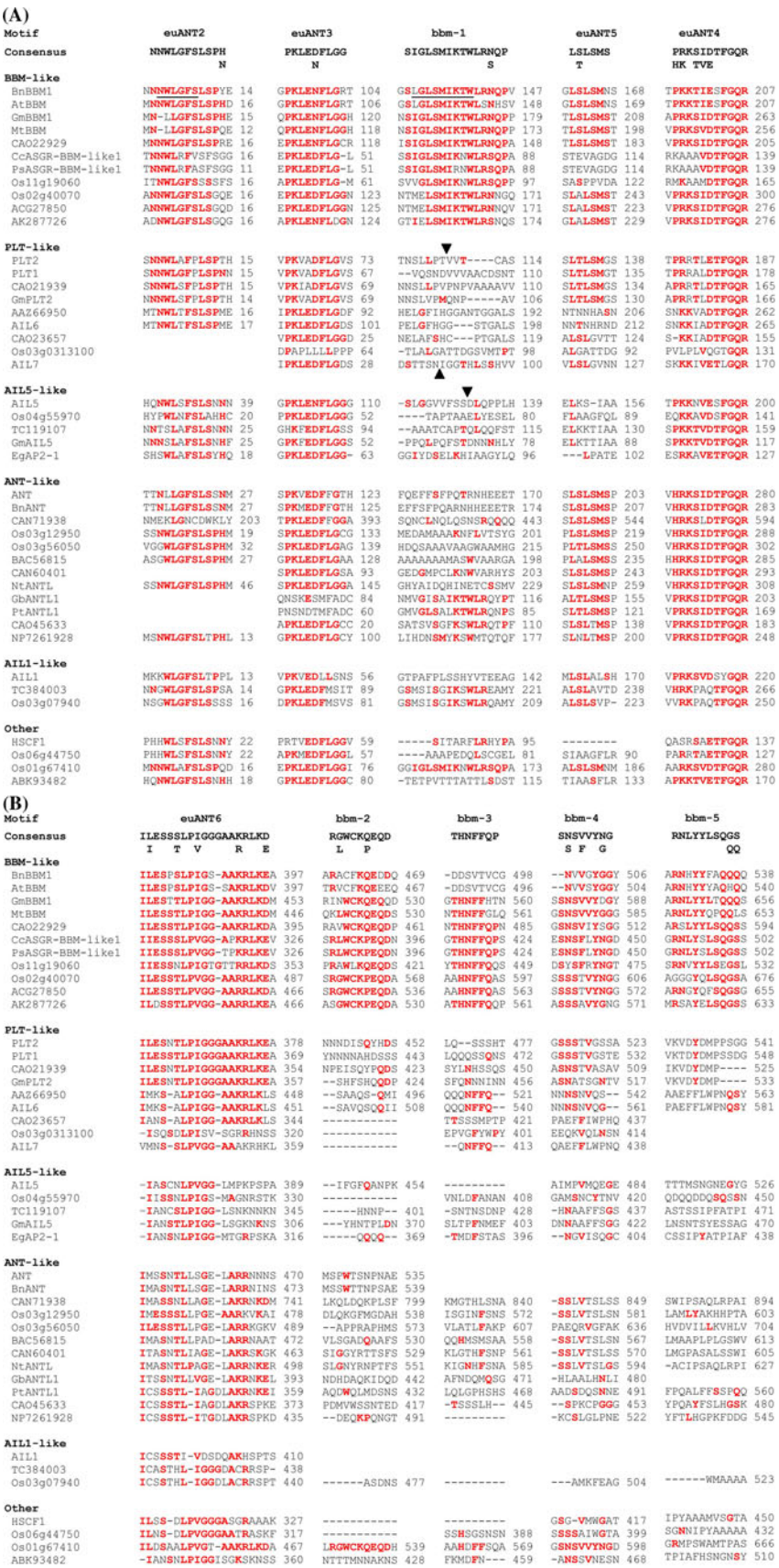

Fig. 2 Motifs in the N-terminal (**A**) or C-terminal (**B**) sequences of the *BBM*-like genes and their conservation in the euANT subgroup of the AP2 family. Residues that match the consensus sequence are in red. Sequences are grouped into clades as indicated in the phylogenetic tree. The sequences of the euANT2 and bbm-1 motifs in the *BnBBM1* sequence that were deleted to test their function are *underlined*. For the domain swap analysis, the site of insertion of the bbm-1 motif from BnBBM1 (SLGLSMIKTWLRNQP) in PLT2, AIL5, and AIL7 are indicated with *arrowheads*

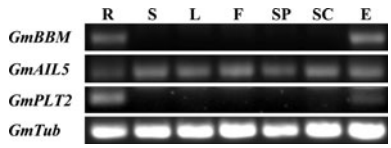

Fig. 3 Accumulation of *GmBBM1*, *GmAIL5*, *GmPLT1* and tubulin (accession number M21297) mRNA in soybean cv Merril genotype X5 detected by RT-PCR. Roots at 1 week (R), stem at 3 weeks (S), leaf at 3 weeks (L), flowers at 2 dpa (F), seed pod at 21 dpa (SP), seed coats at 21 dpa (SC), embryos at 21 dpa (E)

AIL5 homologue and *GmPLT2* as a *PLT* homologue based on the gene phylogeny, conservation of the N-terminal and C-terminal sequences and the expression patterns.

Phenotypes generated by *BBM*-like genes in transgenic *Arabidopsis*

A comparison of the embryogenic phenotype and pleiotropic effects conferred by *GmBBM1* and *BnBBM1* was carried out in transgenic Arabidopsis using the pBINPLUS vector system used previously for ectopic overexpression of *BnBBM1* (Boutilier et al. 2002) (Fig. 4). We confirmed previous descriptions (Boutilier et al. 2002) that showed *BnBBM1* expression in Arabidopsis induced somatic embryos and cotyledon-like structures on post-germination organs of transgenic seedlings as well as a unique set of pleiotropic effects on both the vegetative and reproductive organs (Table 1, BnBBM1 lines 1–3). Approximately 35 independent transgenic Arabidopsis lines expressing *GmBBM1* embryos on the cotyledons were examined and six representative lines were followed into the T2 generations (Fig. 4A–C). Table 1 show the data for 3 of the GmBBM1 lines. Somatic embryogenesis generally occurred at a lower frequency than with *BnBBM1* (Table 1). The highest frequency was 28% in the GmBBM1 line 1, followed by 17% in the GmBBM1 line 2 and 5% in the GmBBM1 line 3 ($n > 100$ for each line). Embryos also emerged from the shoot apical meristem (Fig. 4D) and hypocotyls (Fig. 4E). Pleiotropic effects on development that were typical of *BnBBM1* overexpression were again evident with *GmBBM1* overexpression. These included short roots (80% penetrance, GmBBM1 line3; $n = 100$),

Fig. 4 Phenotypes induced by expression of *GmBBM1* in transgenic Arabidopsis seedlings. Somatic embryos differentiating from the tips of cotyledons (**A**, **B**, **C**). Ectopic roots differentiating from the embryogenic tissues (**A**, **C**). Somatic embryos differentiating from the shoot apical meristem (**D**) and hypocotyls (**E**). Alterations in seedling development characterized by short roots, swollen hypocotyls and elongated cotyledons (**E**, **F**). Un-transformed control seedlings for comparison (**G**)

Table 1 Phenotypes of individual transgenic lines expressing *BnBBM1* and *GmBBM1* constitutively in transgenic Arabidopsis

Description of phenotype	Transgenic BnBBM1 lines			Transgenic GmBBM1 lines		
	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
Somatic embryo formation on cotyledons	++++	++++	+++	++	+	+
Short roots, fibrous roots	+++	++++	+	+	+	++++
Elongated roots	–	–	–	++	+	–
Thick, short hypocotyls	+	+	+	++++	++++	++++
Elongated cotyledons	+	–	++	+	–	+
Delayed flowering time	++++	++++	+++	++	+	+
Reduced seed set	+	+	+	+++	+++	+++
Increased floral organ size	+	+	+	+	++	++

The phenotypes in bold at the seedling stage of development and used for the initial screening of lines. The frequency of occurrence of the phenotype was recorded for each line using at least 100 plantlets

+ (>0<25%), ++ (25–50%), +++ (50–75%), ++++ (75–100%)

short hypocotyls (greater than 80% penetrance in all GmBBM1 lines tested), elongated cotyledons (low penetrance phenotype <25% in all GmBBM1 lines) on seedlings (Fig. 4E, F). Later in development a range of pleiotropic effects were noted including altered leaf morphologies and thickened floral stems. Increased numbers of inflorescences were also noted (data not shown). During the reproductive phase of growth, *GmBBM1* and *BnBBM1* both induced delayed-flowering time; increased floral organ size; thickened and increased numbers of inflorescences; decreased silique size; and decreased seed production (Table 1).

Although many of the phenotypes were similar for *BnBBM1* and *GmBBM1* some interesting differences were observed. For example, in GmBBM1 transgenic lines ectopic roots occassionally developed on cotyledons along with embryos (Fig. 4A, C), a feature that was not reported with BnBBM1 (Boutilier et al. 2002). Furthermore, elongated roots were seen on plantlets of the GmBBM1 line in contrast to the short-root phenotype observed in BnBBM1 plants (Table 1).

Function of the bbm-1 motif in embryogenesis

The significance of the bbm-1 sequence motif to *BnBBM1* function was examined by creating a 9-amino acid deletion mutation in the *BnBBM1* gene (Fig. 2A) and assessing functionality in transgenic Arabidopsis (Fig. 5). For comparison, a deletion of motif euANT2 (Fig. 2A) was also created singly and together with a deletion of bbm-1. The induction of somatic embryos on cotyledons by wild-type *BnBBM1* was first confirmed in 22 transgenic lines as controls using the pCAMBIA1300 vector (Fig. 5C). Shoots subsequently developed in culture (Fig. 5D) and plantlets regenerated as described previously by Boutilier et al. 2002 (data not shown). Somatic embryogenesis was not eliminated by deletion of euANT2 (Fig. 5G, H) or euANT2 and bbm-1 together (Fig. 5I, J). However, the mutant phenotypes differed in that the embryo-like structures tended to proliferate and shoots did not emerge over time (Fig. 5H, J) as with wild-type *BnBBM1* (Fig. 5D). This phenotype was confirmed in 14 and 25 transgenic lines, respectively and was in striking contrast to the phenotype generated by deletion of the bbm-1 motif alone which was characterized by the complete loss of somatic embryogenesis on cotyledons as well as the accompanying pleiotropic effects on seedlings (Fig. 5E, F). Pleiotropic effects associated with this mutation emerged at later stages of plant development. Some were similar to the class II pleiotropic effects described for BnBBM1 by Boutilier et al. (2002). For example, flowers with short sepals and petals relative to carpels were frequently observed (Fig. 6A) compared with wild type flowers (Fig. 6B). The changes in leaf morphology described for BnBBM1 (Boutilier et al. 2002) were infrequently observed; however, serrated margins were occasionally observed (Fig. 6C) in plants expressing the deletion mutant but not wild type plants (Fig. 6D).

The bbm-1 motif was inserted into Arabidopsis PLT2, AIL5 and AIL7 (Fig. 2A) to determine if other euANT members possessed the complementary elements needed for somatic embryogenesis in transgenic Arabidopsis. AIL7 + bbm-1 and AIL5 + bbm-1 failed to generate ectopic embryos in experiments parallel to those above (data not shown). The ectopic PLT2 phenotype was previously characterized by the proliferation of ectopic roots and root hairs on germinating embryos or seedlings (Aida et al. 2004). PLT2 + bbm-1 generated a range of phenotypes in five different transgenic lines. Rapid and strong tissue proliferation followed by copious embryo differentiation was observed in 4 of the 5 lines (Fig. 7A, B). This phenotype resembled the BBM phenotype (Ben Scheres and colleagues, personal communication; Fig. 7). Smaller

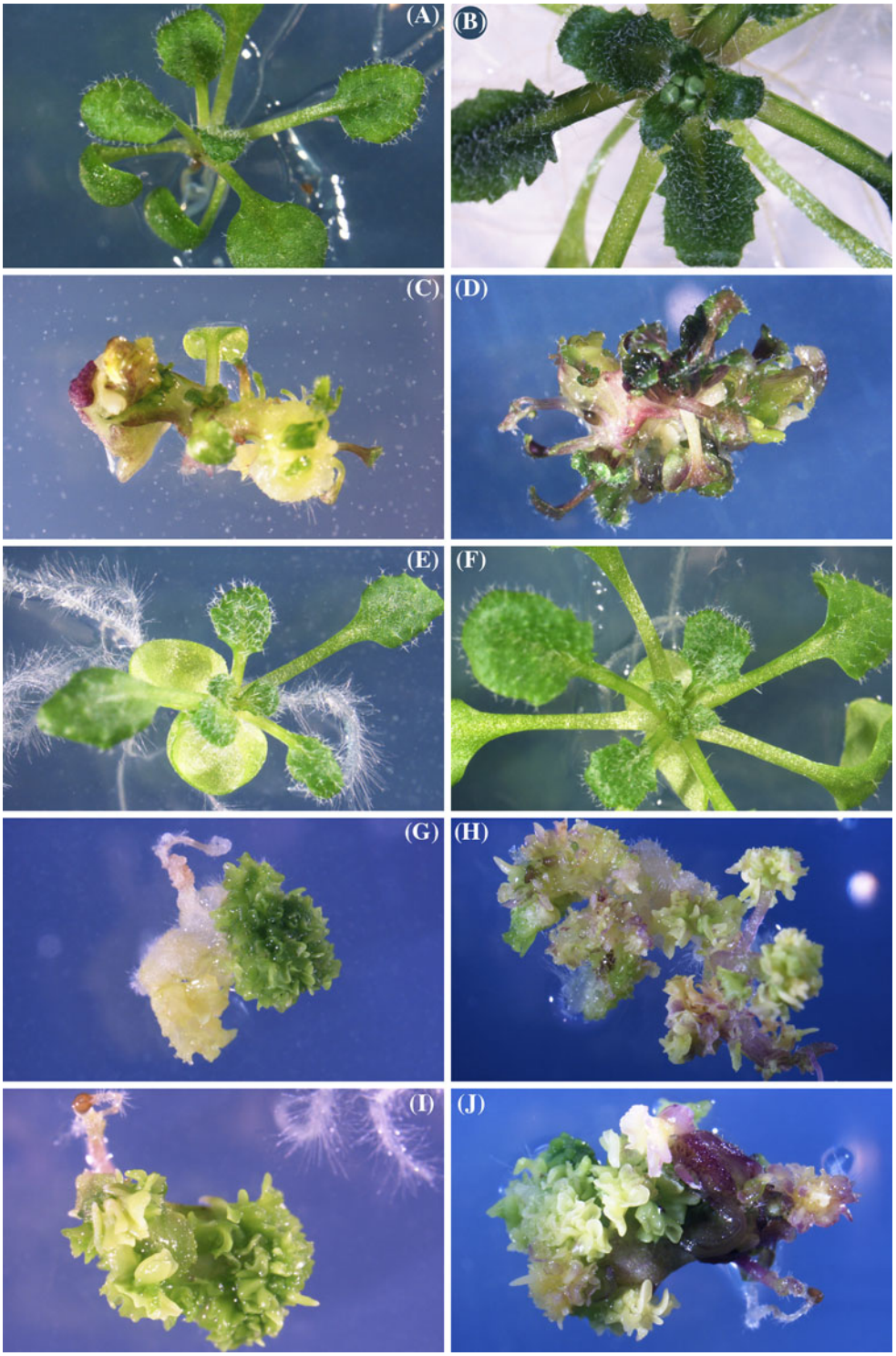

Fig. 5 Phenotype of un-transformed Arabidopsis seedlings at 14 days (**A**) and 21–28 days (**B**) post germination compared with the phenotype of 14 day and 21–28 day transgenic Arabidopsis ectopically expressing wild-type *BnBBM1* (**C**, **D** respectively), the *BnBBM1* deletion mutant without bbm-1 (**E**, **F** respectively), the *BnBBM1* deletion mutant without euANT2 (**G**, **H** respectively), the *BnBBM1* deletion mutant without euANT2 and bbm-1 (**I**, **J** respectively)

undefined green tissues had also been described as part of the PLT2 ectopic phenotype (Aida et al. 2004) In 2 of the lines, both BBM and PLT2 phenotypes were expressed simultaneously. In these lines embryo differentiation was less prolific and root differentiation was very abundant (Fig. 7C, D).

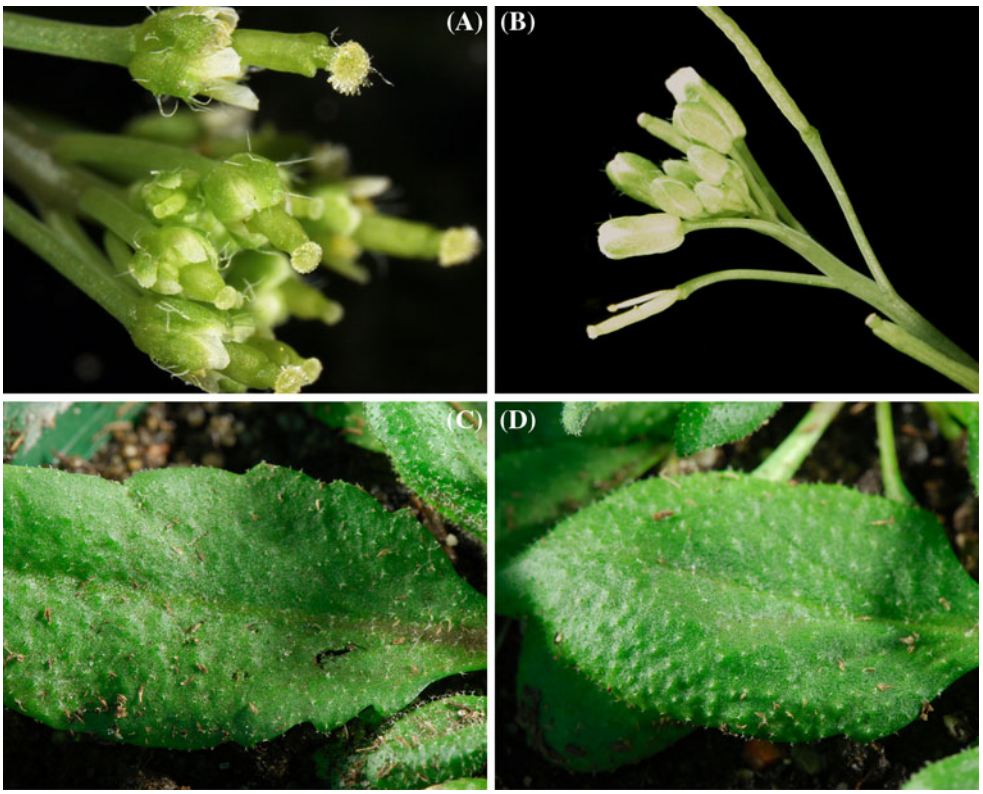

Fig. 6 Pleiotropic effects on transgenic Arabidopsis flower (**A**) and leaf (**C**) morphologies generated by the *BnBBM1* deletion mutant that lacks the bbm-1 motif compared with wild type flowers (**B**) and leaf (**D**)

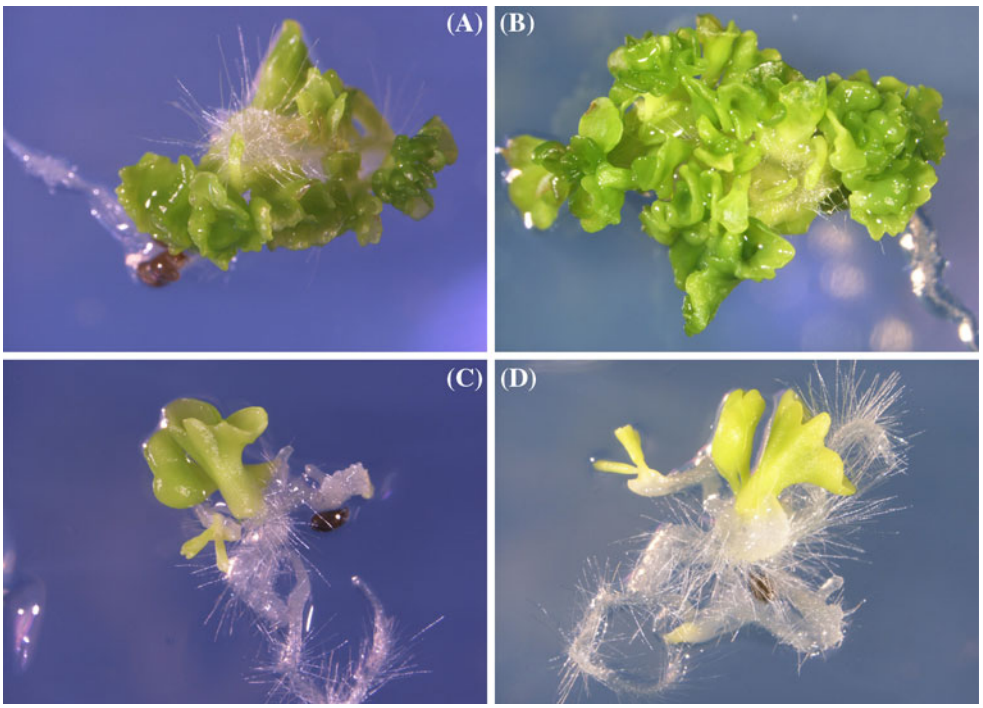

Fig. 7 Phenotypes of transgenic Arabidopsis seedlings, in which the bbm-1 motif from *BnBBM1*was inserted into the corresponding position of the *PLT2* gene, at 11 days (**A**, **B**), 13 days (**C**) and 17 days (**D**) post germination

Discussion

The phenotypes associated with the AP2/ANT family frequently involve undifferentiated cell proliferation and differentiation of stem cell niches within meristems (Nole-Wilson et al. 2005). Recently, a role in embryogenesis has also emerged. Most of the information has been generated by the induction of somatic embryogenesis by ectopic overexpression of *BnBBM1* in Arabidopsis and *B napus* (Boutilier et al. 2002; Passarinho et al. 2008). *GmBBM1* was shown here to induce similar developmental events in Arabidopsis. Compared with meristematic cell types, the differentiation of embryonal stem cells from somatic cells is poorly understood. Studies have shown that somatic embryogenesis may be activated through a number of different pathways and by a number of different genes (Passarinho et al. 2008; reviewed by Verdeil et al. 2007). In this study, we have identified specific features of the *BBM*-like homologues that are responsible for the induction of embryogenesis and that may separate the *BBM*-like genes from closely related members of the AP2/ANT family that function in vegetative development yet act redundantly in embryo development.

To understand how *BBM*-like genes function in the conversion of somatic cells to embryonal cells and the development of the resulting embryo it may be important to understand the relationships among the AP2/ANT family members. The members of the euANT lineage, such as the *BBM*-like, *PLT*-like and *AIL5*-like genes, are very closely related phylogenetically and structurally. Our analysis has revealed domain conservation among them but also domain specificity. For example, the bbm-1 motif was specific to all of the *BBM*-like genes. The different members are known to have specific roles in development but evidence for redundancy is also emerging. For example, the mutant phenotypes of *bbm* and *plt2* have shown that *AtBBM* and *PLT2* function as redundant partners in early Arabidopsis embryogenesis (Galinha et al. 2007). *AtBBM, PLT1, PLT2* and *PLT3/AIL6* are expressed in root primordia where they function as redundant partners in root stem cell differentiation in embryos and root differentiation (Aida et al. 2004; Galinha et al. 2007). The demonstration that the addition of the bbm-1 motif to PLT2 enhanced the capacity to induce somatic embryogenesis confirmed the close relationship between these genes and may have revealed a key structural feature of BBM important for its ability to function in embryogenesis. The PLT2 ectopic phenotype was not lost when the BnBBM1 ectopic phenotype was expressed but the strength of the PLT2 and BnBBM1 ectopic phenotypes were inversely related indicating that the embryogenic and root meristematic pathways could be competing in these transgenic lines.

How *AIL5* functions, particularly in embryogenesis, is unclear. *AIL5* appears to be involved in cell proliferation activities in many organs and may generate enlarged organs, for example large flowers, when ectopically expressed (Nole-Wilson et al. 2005). This phenotype is similar to that of *ANT*, which generates large flowers (Nole-Wilson et al. 2005; Mizukami and Fischer 2000) and *AP2* which generates large embryos (Ohto et al. 2005) when ectopically expressed. Since *AIL5* mutants have no apparent phenotype it is believed that it acts redundantly with other relatives (Nole-Wilson et al. 2005). *AIL5* and *BBM* double mutants appear to lack defects in embryo or root meristem development therefore additional unidentified partners may exist if they act in the same pathways (Tsuwamoto et al. 2010). The finding that ectopic over-expression of *AIL5* yielded phenotypes similar to *BBM* over-expression, including somatic embryogenesis (Tsuwamoto et al. 2010), indicates that it could be involved in the proliferation of the totipotent somatic cells. The embryonic structures that emerged exhibit elevated expression of AGL15, LEC1, LEC2, FUS3 and BBM (Tsuwamoto et al. 2010) all of which have the capacity to induce somatic embryogenesis when ectopically expressed. Interesting, the oil palm *AIL5*-like gene, *EgAp2-1,* is also expressed in proliferating tissues, especially embryonic tissues but induces callus growth and shoot organogenesis rather than embryos in Arabidopsis (Morcillo et al. 2007). *BnBBM1* also induces shoot organogenesis in tobacco rather than embryogenesis revealing that *BBM*-like genes have the capacity to induce both shoot meristem activity as well as embryogenesis depending on the genetic and cellular environment of the proliferating cells (Srinivasan et al. 2007). Unlike *PLT2*, the addition of the bbm-1 motif to *AIL5* did not generate the capacity to induce embryogenesis in transgenic Arabidopsis in this study. Whether this alteration disrupted the capacity of AIL5 to generate somatic embryos as described by Tsuwamoto et al. (2010) is unknown and points to complex roles for AIL5 in plant development.

Conserved sequence motifs have been recognized among soybean, rice and Arabidopsis AP2/ANT genes and soybean-specific motifs are among them (Zhang et al. 2008). Previous studies have suggested that within subfamilies conserved sequence motifs are abundant and likely reflect shared functions (Nakano et al. 2006). Unlike some of the well-characterized motifs of the ERF family, sequence motifs within the AP2/ANT family have not yet been assigned specific roles in the transcriptional regulation of developmental processes (Nakano et al. 2006; Zhang et al. 2008). The characterization of the *GmBBM1* gene here allowed us to align the *BBM*-like genes from divergent species which are known to generate ectopic embryos in Arabidopsis and thus provide a functional data

set that was used to identify 10 conserved sequence motifs outside of the AP2-linker-AP2 region. Among the *BBM*-like, *PLT*-like and *AIL5*-like genes the motif composition was very similar, however, the bbm-1 motif was the most specific to the *BBM*-like genes in the N-terminal region. In the C-terminal region sequences with a lower degree of conservation among the *BBM*-like genes exist. Of these bbm-2, bbm-3 and bbm-4 are poorly conserved in the cruciferous genes and thus unlikely to be essential for embryogenesis. The motif, bbm-5, is also poorly conserved relative to bbm-1.

Both deletion and domain swap analyses revealed that the bbm-1 motif was needed for *BnBBM1* to induce somatic embryogenesis in Arabidopsis. Further examination revealed that bbm-1 activity is intimately linked to the activities of other motifs within these transcription factors, especially euANT2, which is found in almost all other genes in the euANT lineage including PLT2 and AIL5. Deletion of euANT2 singly or euANT2 and bbm-1 simultaneously prevented the somatic embryos from generating shoots even after prolonged times in culture. Among the many possible explanations for bbm-1 function is the possibility that the euANT2 motif functions in vegetative pathways of development and that bbm-1 acts to restrain the vegetative activity of BnBBM1. If correct, then a mechanism must exist to relieve the restraints on euANT2 in BnBBM1 during the later stages of embryo development when the shoot and root meristems differentiate and develop. Furthermore, if a single domain, bbm-1, can separate the ectopic phenotypes of redundant partners such as BBM and PLT2 then it would seem possible that the shared motifs could provide the basis for functional redundancy. A hierarchical process must exist in the cellular environment to recruit and coordinate the different *AP2/ANT* members to the different developmental pathways in an orderly manner. As both the bbm-1 and euANT2 motifs have the consensus sequence for phosphorylation post-translational processes may be involved in the regulation of such interactions. For PLT-like genes, expression gradients appear to play an important in the transition from root meristem initiation to root development (Galinha et al. 2007).

Any general model for the role of *AP2/ANT* transcription factors in somatic embryogenesis should consider the observations that *BBM*-like genes may play distinct roles at different stages of development; interact with redundant partners; and induce different pathways of development depending on the genetic and cellular environment. The bbm-1 motif of *BBM*-like genes appears to play an early role in the conversion of somatic cells to neoplastic embryonal cells. This may occur by suppressing the activity of other motifs, such as euANT2, which function in vegetative cell differentiation. The shared motifs among the AP2/ANT members may provide a mechanism for BBM to recruit the activities of members with meristematic activities essential for the development of the embryo once embryogenesis is established. As new members are recruited to the developing embryo dilution of the bbm-1 motif may release the cells from the neoplastic state and allow the meristems to develop. Sustained ectopic expression of BBM genes during plant maturation results in a range of pleiotropic effects. This may occur through similar unintended interactions with AP2/ANT proteins through shared motifs that disrupt their normal patterns of activity. The different pleiotropic effects observed for GmBBM1 and BnBBM1 may reveal differences in the efficiencies of interactions resulting from sequence divergence. If *BBM*-like genes function by utilizing redundancies with other AP2/ANT transcription factors to recruit developmental pathways needed for embryogenesis and embryo development in seeds then strict spatial control of *BBM*-like gene expression would be essential for normal plant development. As shown here and in all other studies of *BBM*–like gene expression (Boutilier et al. 2002; Nole-Wilson et al. 2005; Imin et al. 2007) expression in seeds is under strict spatial and developmental regulation as are the other AP2/ANT family members (Feng et al. 2005).

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